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# Aflatoxin B1 Transfer and Metabolism in Human Placenta

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**Aflatoxin B1 (AFB1), a common dietary contaminant, is a major risk factor of hepatocellular carcinoma (HCC). Early onset of HCC in some countries in Africa and South-East Asia indicates the importance of early life exposure. Placenta is the primary route for various compounds, both nutrients and toxins, from the mother to the fetal circulation. Furthermore, placenta contains enzymes for xenobiotic metabolism. AFB1, AFB1-metabolites, and AFB1-albumin adducts have been detected in cord blood of babies after maternal exposure during pregnancy. However, the role that the placenta plays in the transfer and metabolism of AFB1 is not clear. In this study, placental transfer and metabolism of AFB1 were investigated in human placental perfusions and in *in vitro* studies. Eight human placentas were perfused with 0.5 or 5μM AFB1 for 2–4 h. *In vitro* incubations with placental microsomal and cytosolic proteins from eight additional placentas were also conducted. Our results from placental perfusions provide the first direct evidence of the actual transfer of AFB1 and its metabolism to aflatoxicol (AFL) by human placenta. *In vitro* incubations with placental cytosolic fraction confirmed the capacity of human placenta to form AFL. AFL was the only metabolite detected in both perfusions and *in vitro* incubations. Since AFL is less mutagenic, but putatively as carcinogenic as AFB1, the formation of AFL may not protect the fetus from the toxicity of AFB1.**

*Key Words:* **human placental perfusion; fetal exposure; placental cytosolic fraction; aflatoxicol; dietary carcinogen.**

Due to immaturity and rapidly developing organs, the fetus may be especially susceptible to the effects of environmental toxins (for recent reviews see Godschalk and Kleinjans, 2008; Neri *et al.*, 2006; Perera *et al.*, 2002). Strong implications exist between the growth and health of a fetus and infant and risk of several diseases later in life (as reviewed by Gluckman *et al.*, 2008; Wild and Kleinjans, 2003). Increasing incidences of immunological disorders and childhood cancer in general and especially links with leukemia and central nervous system tumors have been observed during past decades (Dreifaldt *et al.*, 2004; Pallapies, 2006). The geographical distribution of some cancer types, especially hepatocellular carcinoma (HCC), is unevenly distributed. In sub-Saharan Africa and South-East Asia where 80% of HCC cases occur, there is a higher prevalence in children than seen in other regions (IARC, 2003). It is thought that besides genetic factors, exposure to environmental agents during pregnancy and in early life plays a role in the development of immunological disorders and childhood cancer (Bunin, 2004; Gluckman *et al.*, 2008; Pallapies, 2006).

Aflatoxin B1 (AFB1) is a fungal toxin produced by a species of *Aspergillus*, mainly by *Aspergillus flavus*, and is a common food contaminant all over the world, mostly in the regions where hot and humid climates favor the growth of these fungi and where food is improperly stored (Williams *et al.*, 2004). Several lines of evidence point to a significant exposure to AFB1 both at prenatal and postnatal stages. First, several studies have reported high levels of aflatoxins in maternal and cord blood of mothers living in contaminated areas, which proves that aflatoxins can cross human placenta (Abdulrazzaq *et al.*, 2002; Denning *et al.*, 1990; Hsieh and Hsieh, 1993). Second, aflatoxin-albumin (AF-alb) adducts have been detected both in maternal and in cord blood (Turner *et al.*, 2007; Wild *et al.*, 1991). Third, in infants and young children, AF-alb adducts have been found in blood (Gong *et al.*, 2004; Turner *et al.*, 2007) and aflatoxins present in urine (Polychronaki *et al.*, 2008). As to the health effects, AFB1 is associated with growth impairment (Gong *et al.*, 2004; Turner *et al.*, 2007) and reduced IgA levels in children (Turner *et al.*, 2003). Considering that AFB1 is a major etiological factor in the development of the HCC (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002), it is anticipated that exposure to AFB1 *in utero* and in early stages of infant life would contribute to the early onset of HCC in countries with a high incidence of the disease.

AFB1 like most carcinogens requires metabolic activation to elicit its toxic properties. It is metabolized by CYP1A2 and 3A

The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

(3A4, 3A5, and 3A7) enzymes into several metabolites, which are either secreted out of the body or can react with cellular macromolecules like DNA and proteins (Eaton and Gallagher, 1994; Guengerich *et al.*, 1998; IARC, 2003). In adult liver, CYP1A2 and 3A4 are the main enzymes catalyzing AFB1 metabolism (Gallagher *et al.*, 1994; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002), while in human prenatal liver, CYP3A7 plays a major role in AFB1 metabolism (Doi *et al.*, 2002; Kitada and Kamataki, 1994). The best-known metabolite is AFB-8,9-epoxide, the formation of which is mainly responsible for the carcinogenicity of AFB1. AFB1 can also be metabolized by NADPH-dependent reductase into a carcinogenic metabolite aflatoxicol (AFL) (Salhab and Edwards, 1977; Wong and Hsieh, 1976). AFL acts as a reservoir of AFB1, prolonging its lifetime in body, as it can be reconverted to AFB1, which then can be further metabolized. Formation of AFL does not decrease the toxicity of AFB1 because it can also bind to DNA and is as potent carcinogen like AFB1 (Bailey *et al.*, 1994, 1998).

The placenta contains low levels of CYP enzymes, mainly CYP1A1, reductases, and transferases with variation depending on the stage of placental development (Hakkola *et al.*, 1996a,b; Pasanen, 1999; Pavek and Dvorak, 2008). CYP1A2 has been detected at messenger RNA (mRNA) level in the placenta during the first trimester, although no functional activity has been detected during this time or in term placentas (Hakkola *et al.*, 1996a,b; Myllynen *et al.*, 2007). CYP3A4–7 enzymes have been detected both at mRNA and at protein levels in first trimester and term placentas, but these enzymes are not functionally active (Hakkola *et al.*, 1996a,b; Myllynen *et al.*, 2007). These findings indicate that human placenta may not be capable of CYP-mediated activation of AFB1. On the other hand, Datta and Kulkarni (1994) have reported that lipoxygenase (LO) in human term placenta, and in the intra uterine conceptal tissue (including placenta) at 8–10 weeks of gestation, is capable of epoxidation of AFB1 (Datta and Kulkarni, 1994). Increase of mutagenic activity of AFB1 in *Salmonella typhimurium* TA100 by placental microsomal protein is another indication of the capacity of placental tissue to activate AFB1 (Sawada *et al.*, 1993). These limited findings clearly indicate that the placental metabolism of AFB1 with potential detrimental consequences to the fetus and placenta itself is worth further scrutiny. Consequently, the primary target of this paper was AFB1 metabolism and transfer in human placenta using human placental perfusion and *in vitro* incubations with placental tissue fractions.

## MATERIALS AND METHODS

### *Chemicals*

Aflatoxin B, AFL, and NADPH were purchased from Sigma (St Louis, MO). $^{3}$H-labeled AFB1 was purchased from American Radiolabeled Chemicals (St Louis, MO). All other chemicals and reagents were of the highest quality commercially available.

### *Nuclear Magnetic Resonance Spectroscopy*

The identity of AFL reference was confirmed by liquid-state nuclear magnetic resonance (NMR) spectroscopy. Liquid-state NMR spectra were recorded in $CD_3CN$ using a Bruker AV 500 spectrometer (500.1 MHz for $^{1}$H), referenced to the residual protonated solvent peak (1.94 ppm).

### *Collection of Placentas*

In Finland, human placenta is discarded after delivery and the use of the placenta for research purposes does not affect the delivery or the treatment of the mother and child in any way. The Research Ethics Committee of the University Hospital District of Kuopio region reviewed and approved this study on 11 May 2005. Placentas were collected immediately after normal delivery or caesarean section from uncomplicated fulltime pregnancies of healthy nonsmoking mothers who delivered in Kuopio University Hospital. All mothers were informed about the study by a nurse and also written information was given before a written consent was obtained. Placentas were anonymized for the study.

### *Placental Perfusion*

Placental perfusion was conducted essentially as described earlier (Myllynen *et al.*, 2008; Pienimaki *et al.*, 1995). Krebs-Ringer phosphate buffer with heparin was injected into the umbilical cord vessels within 10 min of the birth of the placenta. The chorionic artery and vein were cannulated from the intact peripheral cotyledon area, and the lobule was placed into the perfusion apparatus. In the maternal side, two cannulas were inserted through the basal plate into the intervillous space of the placenta.

Both the maternal and the fetal sides of the placenta were perfused separately. The perfusate consisted of Roswell Park Memorial Institute 1640 cell culture medium with penicillin-streptomycin (25 U/ml), L-glutamine (2mM), sodium pyruvate (1mM), nonessential amino acid solution (10 ml/l), dextran (2 g/l), heparin (25 IU/ml), and human albumin (2 g/l). The final maternal volume was 200 ml, and the final fetal volume was 100 ml. Perfusion flow rate was 3 ml/min on the fetal side and 9 ml/min on the maternal side. During the perfusion, physiological conditions (37°C and pH 7.4) were maintained as closely as possible. The perfusate was gassed with a mixture of nitrogen and carbon dioxide (95% $N_2$ + 5% $CO_2$) in the fetal circulation and a mixture of oxygen and carbon dioxide (95% $O_2$ + 5% $CO_2$) in the maternal circulation.

The placenta was preperfused for at least 30 min to allow the placenta to recover from hypoxia. If the perfusion was stable (leak from fetal to maternal side less than 3 ml/h, pH within physiological range), AFB1 (0.5 or 5μM) and antipyrine (100 μg/ml) were added to the maternal perfusate and the perfusion was conducted for 2 or 4 h. Antipyrine, which goes through the placenta by passive diffusion, was used as a reference compound to confirm overlap of maternal and fetal cotyledons and thus ensure that circulations on both sides match (Bassily *et al.*, 1995; Brandes *et al.*, 1983; Schneider *et al.*, 1972). Four placentas were perfused with 0.5μM AFB for 4 h and four placentas with 5μM AFB for 2 h. One of the 2-h perfusion was conducted with $^{3}$H-labeled AFB1. Control samples for aflatoxin and antipyrine analysis were taken from both the maternal and the fetal reservoirs before the start of the perfusion. During the perfusions, samples were taken at 5 min, then every half an hour during first 2 h, and once in an hour thereafter. Perfused placental tissue and control samples from the same placenta were collected into 10% neutral formalin for tissue histology and into liquid nitrogen for cell fractionation and DNA purification. Samples were stored at room temperature (formalin samples) or frozen at −80°C. Criteria for valid perfusion were leak from fetal to maternal circulation less than 4 ml/h and previously known transfer of the control compound antipyrine (Myllynen *et al.*, 2008; Pienimaki *et al.*, 1995). Glucose consumption and human chorionic gonadotropin (hCG) hormone production were measured to evaluate viability of placental tissue during perfusion. Glucose, hCG, and leak are shown in Table 1.

### *High-Performance Liquid Chromatography Analysis of Perfusion Samples*

***Aflatoxins.*** Aflatoxins were analyzed immediately after sampling. Perfusate was filtered through 0.45-μm syringe filters (MINISART RC4, Sartorius stedim biotech [GmbH], Goettingen, Germany) and analyzed by high-performance

**TABLE 1**
**Human Placental Perfusion Conducted. Leak Under 4 ml/h from Fetal to Maternal Circulation, Glucose Consumption, and the Production of hCG Indicate Functional Placental Tissue**

| Perfusion number | Duration (h) | Concentration (μM AFB1) | Leak ml/h | Glucose consumption μmol/h/g | hCG mI/ml/g |
|---|---|---|---|---|---|
| 1 | 2 | 5 | 2.8 | NA | 12.8 |
| 2 | 2 | 5 | 2.8 | 59.7 | 20.6 |
| 3 | 2 | 5 | 3.0 | 37.9 | 25.1 |
| 4 | 2 | 5[a] | 3.0 | 23.0 | 6.2 |
| 5 | 4 | 0.5 | 3.4 | NA | 137.6 |
| 6 | 4 | 0.5 | 3.6 | 9.7 | 148.0 |
| 7 | 4 | 0.5 | 2.1 | 3.2 | 17.5 |
| 8 | 4 | 0.5 | 2.1 | 12.5 | 32.0 |

*Note*. NA, not analyzed.
[a]Perfused with $^3$H-AFB1.

liquid chromatography (HPLC; Shimadzu, Kyoto, Japan). The sample (10 μl) was injected into an ODS Speri-5 Brownlee column (5 μm, PerkinElmer, Norwalk, CT) fitted with a $C_{18}$ guard column. The assay was performed at 40°C, the flow rate was 1 ml/min, and the mobile phase used was water-acetonitrile-methanol (60:30:10). All peaks were detected by fluorescence detector (fluorescence detector RF-10AXL; Shimadzu) at 363 nm and quantified by Class VP 6.14 software. The retention time of the AFB1 peak was 7.5 min and the AFL peak was 8.9 min (Fig. 1A). AFB1 and AFL concentrations in perfusate were calculated by comparing the area of the peak with those of standards. Detection limit of this method for AFB and AFL was 0.001μM.

***Antipyrine.*** The samples for analysis of the reference compound antipyrine were stored at −20°C and prepared before analysis. Antipyrine concentrations in perfusion medium were analyzed by an HPLC-ultraviolet (UV) method modified from Myllynen *et al.* (2003). An equal amount of methanol was added to 100 μl of sample and centrifuged (12,000 × g, 15 min). An equal amount of acetonitrile was then added to 150 μl of supernatant, centrifuged once again, and 10 μl of supernatant was analyzed by HPLC (Shimadzu) using Supelco C-14 (250 × 2.5 mm; 5 μm) column. The assay was performed at 40°C with a flow rate of 1 ml/min and a mobile phase consisting of 20mM $KH_2PO_4$ in $H_2O$-acetonitrile (70:30). All peaks were detected by UV detector (Shimadzu) at 255 nm and quantified by LC Solution software. Retention time of the antipyrine peak was 4.8 min. Antipyrine concentration in the perfusate was calculated by comparing the area of the peak with those of standards. Sensitivity of this method was 0.1 μg/ml.

*Tissue and DNA Binding of Aflatoxin*

One perfusion was carried out using $^3$H-labeled aflatoxin to analyze tissue and DNA binding. A piece of placental tissue from one perfusion with $^3$H-labeled AFB1 (American Radiolabeled Chemicals) was homogenized into the buffer (100mM NaCl; 50mM Tris, pH 8; 1% SDS; and 10mM EDTA). A 100 μl sample of tissue suspension was taken, 900 μl of scintillation HiSafe3-liquid (PerkinElmer) was added, and radioactivity was measured by liquid scintillation counting (Wallac 1450 MicroBeta Trilux, PerkinElmer, Turku, Finland).

DNA was isolated from the same tissue sample using a phenol extraction ethanol—precipitation method earlier described by Vähäkangas *et al.* (1985). Scintillation cocktail (1400 μl) was added directly into 750 μg of DNA, and the radioactivity of purified placental DNA was analyzed by scintillation counting.

*Histological Evaluation of Perfused Placental Tissue*

Placental tissue samples were fixed in neutral 10% formalin, embedded in paraffin, and cut into 5-μm sections. After deparaffinization, slides were stained with hematoxylin and eosin.

*Preparation of Tissue Fractions*

Microsomal and cytosolic fractions were prepared by homogenizing pieces of placental tissue into four times volume of buffer (0.1M Tris-HCl and 1mM $K_2$-EDTA, pH 7.4). The homogenate was centrifuged (10,000 × g, 30 min, 4°C) and the supernatant was collected and centrifuged (100,000 × g, 1 h, 4°C) again. Microsomal pellet was homogenized into the buffer solution, and the samples were stored at −80°C. Cytosolic fractions were also collected and stored at −80°C. Protein concentration was measured by using bovine serum albumin (Sigma) as a standard.

*Incubations*

Preliminary incubations were performed by using different protein concentrations and several time points to determine the best conditions for incubations. In final incubations, 1 mg of placental microsomal or cytosolic protein was added to the reaction mixture, which consisted of phosphate buffer (pH 6.8), 5mM magnesium chloride, and 2mM NADPH. The concentration of aflatoxin was 5μM in microsomal incubations and 0.5 or 5μM in cytosolic incubations. Samples were incubated for 1 h at 37°C. The reaction was terminated by heat (94°C, 5 min) and samples were centrifuged (16,000 × g, 20 min, 4°C). The supernatant was collected and filtered using 0.45-μm syringe filters (Sartorius RC4), and peaks were analyzed by HPLC fluorescence detector (fluorescence detector RF-10AXL; Shimadzu) at 363 nm and quantified by Class VP 6.14 software. The retention time of AFB1 peak was 20.6 min and AFL peak 31.8 min (Fig. 1B). Twenty micromolars of ammonium acetate buffer with acetonitrile and methanol (70:15:15) was used as an eluent. The flow rate was 1.5 ml/min, and the assay was performed at 40°C. This method was selected to analyze the incubation samples because the background and separation of peaks was better than with that for perfusion samples. AFB1 and AFL concentrations in incubation samples were calculated by comparing the area of the peak with those of standards.

*Statistics*

Results are expressed as a mean ± SD. Percent formation of AFB1 and AFL was calculated by comparing the amount of AFB1 or AFL at all time points to the measured amount of AFB1 at the beginning of perfusion. Percent change of antipyrine was calculated in the same way. Placental transfer variables were analyzed from repeated measurements utilizing two-way ANOVA with Bonferroni *post hoc* test. The $p$ values less than 0.05 were taken as statistically significant.

## RESULTS

### NMR Spectroscopy

The identity of AFL reference was confirmed by liquid-state NMR spectroscopy. $^1$H NMR δ: 6.79 (doublet [d], 1H, $J = 7.14$ Hz, $H_1$), 6.53 (doublet of doublets [dd], 1H, $J = 2.1$ Hz, 2.8 Hz, $H_2$), 6.50 (singlet [s], 1H, $H_3$), 5.43 (dd, 1H, $J = 2.4$ Hz, 2.8 Hz, $H_4$), 5.1 (multiplet [m], 1H, $H_5$), 4.76 (m, 1H, $H_6$), 3.86 (s, 3H, $H_7$), 3.32 (m, 1H, $H_8$), 3.16 (m, 1H, $H_9$), 3.12 (d, 1H, $J = 5.4$ Hz, 1H, $H_{10}$), 2.33 (m, 1H, $H_{11}$), and 1.88 (m, 1H, $H_{12}$) (Fig. 2).

### Placental Transfer and Metabolism of AFB1

To study the transfer and metabolism of AFB1 through human placenta, AFB1 was added with the reference compound antipyrine into the maternal circulation of perfused placenta. AFB1 kinetics differed from that of antipyrine, which pass placenta mainly by passive diffusion. Both AFB1 and antipyrine crossed the placental barrier quickly and were detected in fetal circulation already 5 min after the addition of AFB1 into the maternal circulation (Figs. 3A and 3B, Tables 2 and 3). When perfused with 0.5μM AFB1, we were able to detect AFB1 in fetal circulation at 5

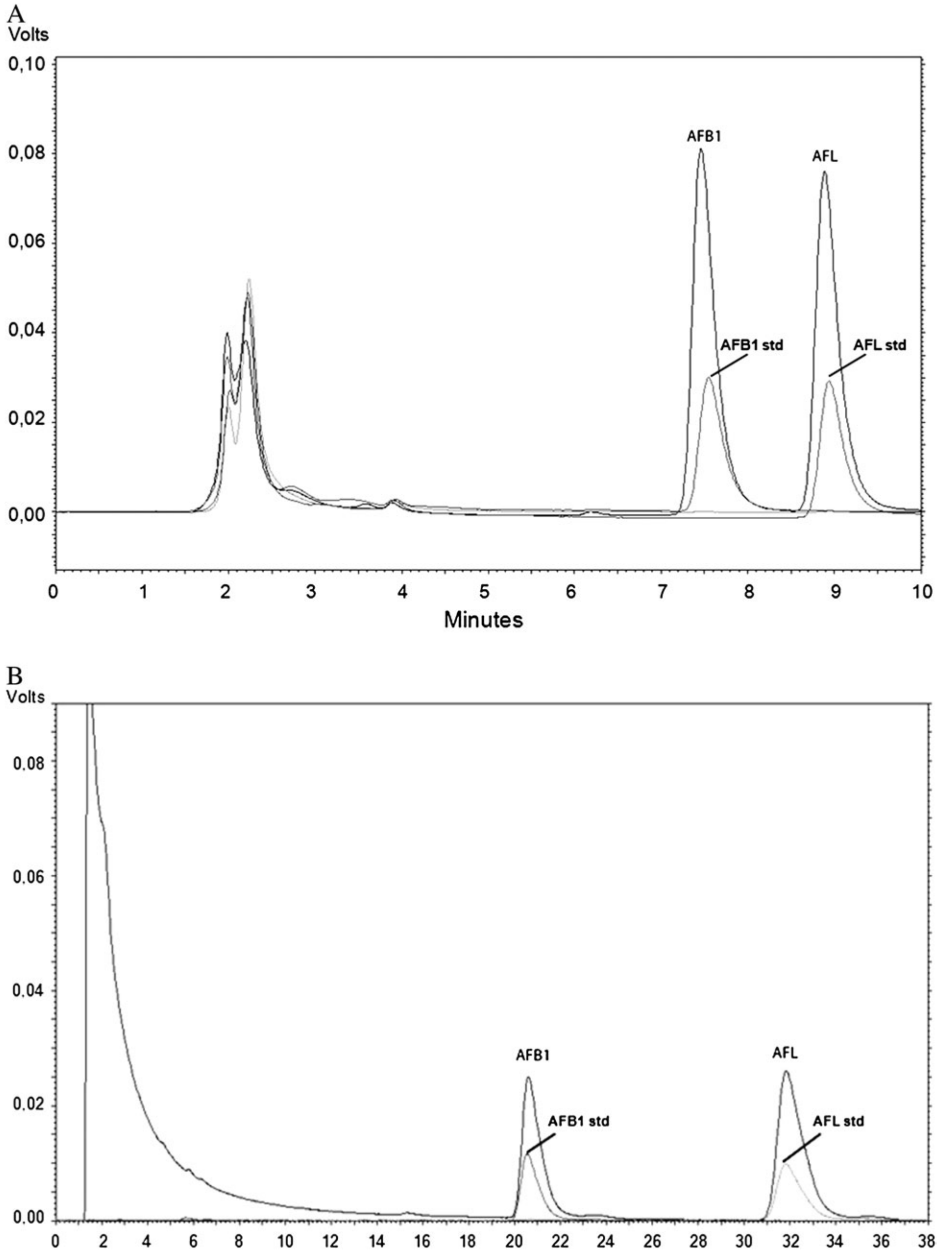


**FIG. 1.** HPLC chromatograms of AFB1 and AFL. (A) Figure represents the chromatogram of the sample from maternal perfusate after a 2-h perfusion with 5μM AFB1 (big peaks), AFB1 standard (small peak at 7.5 min), AFL standard (small peak at 8.9 min), and control sample from maternal circulation before the addition of AFB1. (B) Figure represents of the chromatogram of the sample from incubation for 1 hour with 5 μM AFB1 (big peaks), AFB1 standard (small peak at 20.6 min), AFL standard (small peak at 31.8 min).

min only from one perfusion of four, but when perfused with 5μM AFB1, it was detected from three of four perfusions.

The amount of AFB1 increased in fetal circulation and decreased in maternal circulation over time, but in contrast to the reference compound antipyrine, the concentrations did not equilibrate between fetal and maternal circulation during perfusions. This was due to the metabolism of AFB1 to AFL and the accumulation of AFL in the maternal and fetal circulations (Fig. 3C). The feto-maternal ratio (FM ratio) of AFB1 + AFL remained lower than that of antipyrine (Fig. 4A). This difference was gained earlier with higher concentration (Fig. 4B).

Although an HPLC method used was originally developed for a complete analysis of AFB1 metabolism, apart from AFL no other metabolites were detectable in the perfusion media. AFL was detected from both maternal and fetal circulations as quickly as 5 min after the addition of AFB1 into the maternal perfusate. The amount of AFL increased over time in both circulations, but after 1 h, there was more AFL in the fetal circulation than in the

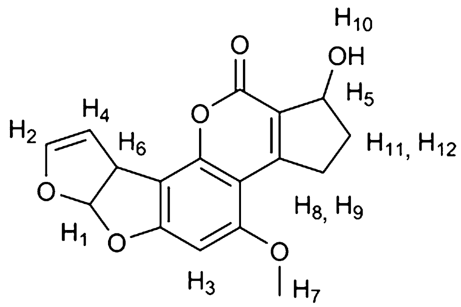


**FIG. 2.** Structure of AFL.

maternal circulation (Fig. 3C). The variation in the formation of AFL between placentas as seen in Figure 3C and Table 4 did not correlate with the size of the perfused placental lobule, indicating interindividual variation in the metabolism. Furthermore, the same amount of cytosolic protein was used in *in vitro* incubations and a sixfold variations were seen. We are confident that variation between placentas is true.

To confirm that no other metabolites were formed, *in vitro* incubations were conducted. Because microsomal fraction (broken smooth endoplasmic reticulum) contains CYP enzymes and cytosolic fraction contains reductases and transferases, incubations were conducted in both fractions to further confirm placental metabolism of AFB1. During incubations, AFL was the only metabolite detected regardless of the cell fractions used. Formation of AFL was much higher in cytosolic fraction. There was about sixfold variation among the eight placentas in the formation of AFL detected with both AFB1 concentrations (0.5 and 5μM) (Fig. 5).

### *Tissue and DNA Binding of AFB1*

To study putative accumulation and DNA binding of AFB1 in placental tissue during the perfusion, one perfusion was carried out with $^{3}$H-labeled AFB1. After the 2-h perfusion with 5μM $^{3}$H-AFB1, 3% of AFB1 was found in homogenized placental tissue. No radioactivity was found in the isolated DNA, indicating very low or nonexistent adduct formation (data not shown). AFL was formed during the perfusion and found in both fetal and maternal circulation, as in all the other perfusions, indicating functioning placental tissue.

### *Histology of Perfused Placental Tissue*

Histological evaluation of the placental tissues showed that perfusion cleared red blood cells from perfused placentas. After perfusions, placental structure with defined villi was preserved (Fig. 6), which indicates good survival of the tissue through both the 2- and the 4-h perfusions. Although AFB1 is toxic to tissues, no overt toxicity was seen in the tissue structure in placentas perfused even with the higher dose (5μM).

## DISCUSSION

The amount of AFB1 (0.5 or 5μM corresponding to 31 or 312 μg/perfusion, respectively) used in this study in perfusions and in

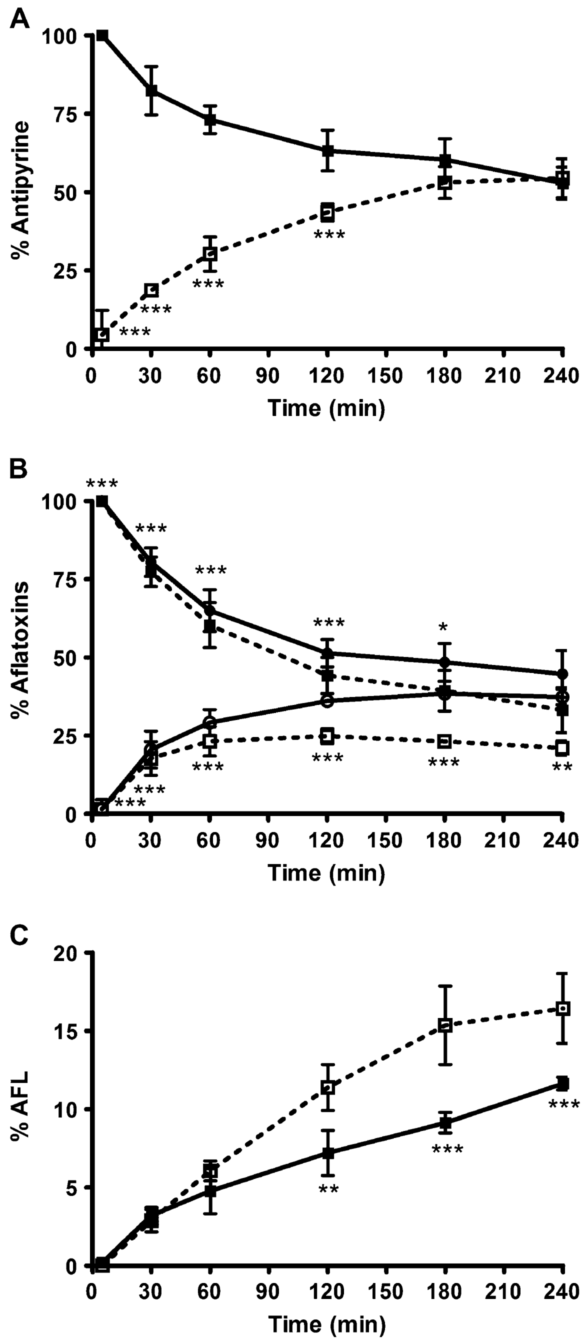


**FIG. 3.** Placental transfer of AFB1 and metabolism to AFL in human placental perfusion. AFB1 (0.5μM) and the reference compound antipyrine (100 μg/ml) were added to the maternal circulation at the beginning of the 4-h perfusions and samples were collected from both the maternal and the fetal circulations at the indicated time points. All values are % of the original concentration analyzed after the addition of the compound, either aflatoxin or antipyrine, into the maternal circulation. (A) Antipyrine in maternal (solid line, filled squares) and in fetal (dotted line, open squares) circulations. (B) AFB1 (dotted line) and AFB1 + AFL (solid line) in maternal (filled symbols) and fetal (open symbols) circulations. (C) AFL in maternal (solid line, filled squares) and in fetal (dotted line, open squares) circulations. Maternal data are compared with the corresponding fetal data at the same time point (mean ± SD, $n = 4$); *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (two-way ANOVA followed by Bonferroni *post hoc* test).

**TABLE 2**
**Concentration of AFB1 in Maternal and Fetal Perfusate at Different Time Points. Mean ± SD Is Given ($n$ = 4)**

| | 2-h perfusions with 5μM of AFB1 ($n$ = 4), AFB1 μg/ml | | 4-h perfusions with 0.5μM of AFB1 ($n$ = 4), AFB1 μg/ml | |
|---|---|---|---|---|
| | Mean ± SD | | Mean ± SD | |
| Time (min) | Maternal | Fetal | Maternal | Fetal |
| 5 | 4.61 ± 1.03 | 0.03 ± 0.03 | 0.77 ± 0.13 | 0.01 ± 0.03 |
| 30 | 3.18 ± 0.53 | 0.68 ± 0.26 | 0.59 ± 0.09 | 0.14 ± 0.06 |
| 60 | 2.34 ± 0.36 | 1.00 ± 0.37 | 0.46 ± 0.08 | 0.18 ± 0.06 |
| 90 | 1.92 ± 0.26 | 1.01 ± 0.29 | — | — |
| 120 | 1.68 ± 0.16 | 0.99 ± 0.27 | 0.33 ± 0.02 | 0.19 ± 0.04 |
| 180 | — | — | 0.30 ± 0.03 | 0.18 ± 0.04 |
| 240 | — | — | 0.25 ± 0.03 | 0.16 ± 0.04 |

*in vitro* incubations reflects the real exposure to AFB1 in some areas of the world. Although in European Union countries the exposure is very low (2–77 ng/person/day) due to efficient control of imported food products (23), in highly contaminated countries like China, the estimated intake of AFB1 ranges from 0 to 91 μg/kg body weight per day, so the estimated daily intake of a 70 kg human in these areas can be as high as 6.4 mg/day (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002).

In pregnant women, high levels of aflatoxins (Denning *et al.*, 1990; De Vries *et al.*, 1989), aflatoxin-DNA adducts (Hsieh and Hsieh, 1993), and aflatoxin-Alb adducts (Turner *et al.*, 2007; Wild *et al.*, 1991) have been detected both in maternal blood and in cord blood showing fetal exposure to aflatoxin *in vivo*. This is the first study ever, where AFB1 transfer and metabolism have been studied in human placental perfusion, a method that retains the structure and function of the tissue (Di Santo *et al.*, 2003). Our results provide direct evidence of a fast transfer of AFB1 through human placenta. However, metabolism of AFB1 to AFL has never been

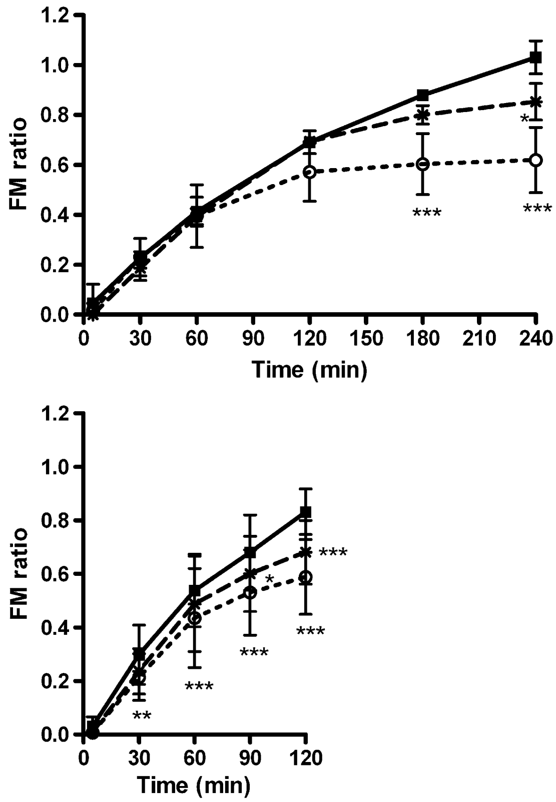


**FIG. 4.** Comparison of FM ratios of AFB1 (circle), AFB1 + AFL (star) and antipyrine (square). (A) In 4-h perfusions with 0.5μM AFB1. (B) In 2-h perfusions with 5μM AFB1. Data are given as a mean ± SD ($n$ = 4). F/M ratios of AFB1 were compared to that of antipyrine; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ (two-way ANOVA followed by Bonferroni *post hoc* test).

**TABLE 3**
**Concentration of Antipyrine (AP) in Maternal and Fetal Perfusate at Different Time Points. Mean ± SD Is Given ($n$ = 4)**

| | 2-h perfusions with 5μM of AFB1 ($n$ = 4), AP μg/ml | | 4-h perfusions with 0.5μM of AFB1 ($n$ = 4), AP μg/ml | |
|---|---|---|---|---|
| | Mean ± SD | | Mean ± SD | |
| Time (min) | Maternal | Fetal | Maternal | Fetal |
| 5 | 88.68 ± 15.07 | 2.36 ± 1.74 | 87.15 ± 21.55 | 3.67 ± 6.57 |
| 30 | 75.14 ± 23.28 | 22 ± 1.87 | 74.17 ± 19.25 | 16.45 ± 2.82 |
| 60 | 67.73 ± 22.17 | 39.36 ± 7.06 | 70.42 ± 19.27 | 25.19 ± 5.97 |
| 90 | 60.19 ± 18.86 | 42.20 ± 6.70 | — | — |
| 120 | 54.24 ± 15.78 | 46.13 ± 7.40 | 64.06 ± 19.29 | 42.04 ± 6.57 |
| 180 | — | — | 55.31 ± 10.06 | 48.42 ± 10.95 |
| 240 | — | — | 46.03 ± 11.06 | 48.36 ± 14.51 |

**TABLE 4**
**Concentration of AFL in Maternal and Fetal Perfusate at Different Time Points. Mean ± SD Is Given ($n$ = 4)**

| | 2-h perfusions with 5μM of AFB1 ($n$ = 4), AFL μg/ml | | 4-h perfusions with 0.5μM of AFB1 ($n$ = 4), AFL μg/ml | |
|---|---|---|---|---|
| | Mean ± SD | | Mean ± SD | |
| Time (min) | Maternal | Fetal | Maternal | Fetal |
| 5 | 0.01 ± 0.01 | 0.01 ± 0.02 | 0.00 ± 0.00 | 0.00 ± 0.01 |
| 30 | 0.14 ± 0.05 | 0.10 ± 0.02 | 0.03 ± 0.01 | 0.02 ± 0.01 |
| 60 | 0.24 ± 0.10 | 0.24 ± 0.05 | 0.04 ± 0.02 | 0.05 ± 0.01 |
| 90 | 0.33 ± 0.14 | 0.33 ± 0.07 | — | — |
| 120 | 0.38 ± 0.18 | 0.41 ± 0.10 | 0.06 ± 0.02 | 0.09 ± 0.02 |
| 180 | — | — | 0.07 ± 0.01 | 0.12 ± 0.01 |
| 240 | — | — | 0.09 ± 0.01 | 0.12 ± 0.02 |

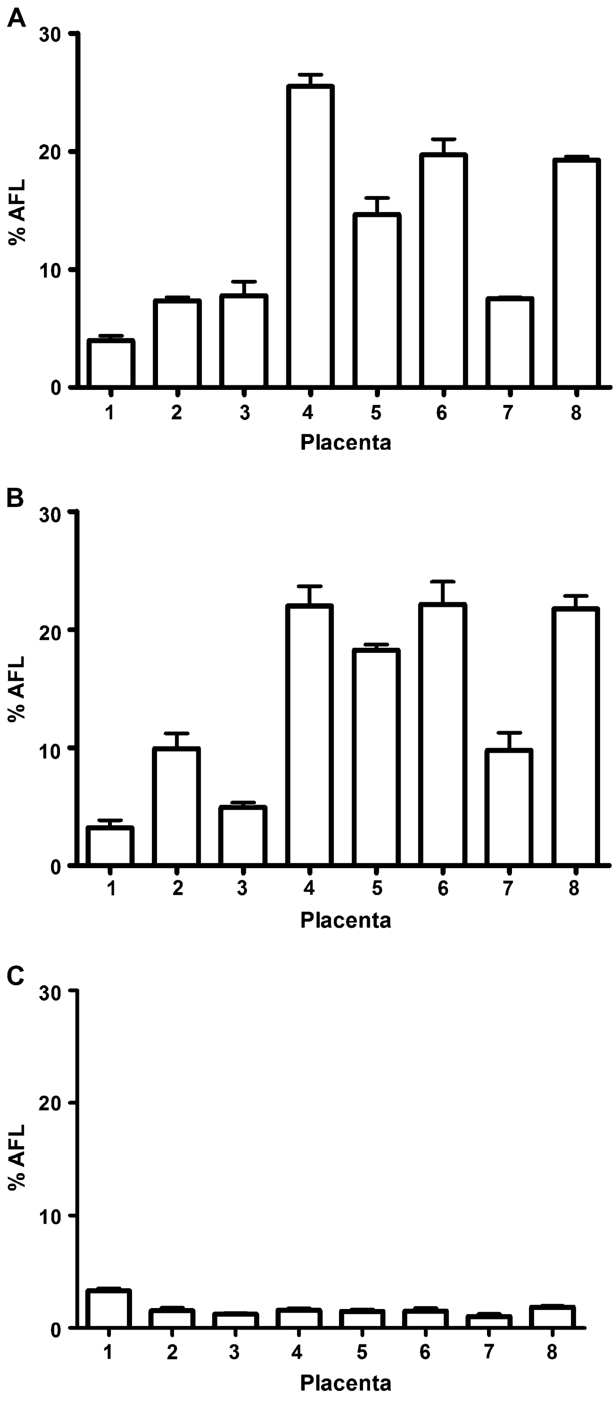


**FIG. 5.** Formation of AFL during 1-h incubations with 0.5μM AFB1 (A) or with 5μM AFB1 (B) with placental cytosolic protein or with 5μM AFB1 with microsomal preparations from the same placentas (C). Data are given as percent from the added amount of AFB1. Every bar represents a mean of three separate incubations using cytosol or microsomes from the same placenta (mean ± SD).

demonstrated in human placenta before. Metabolism to AFL cannot be regarded as a protective mechanism considering fetal health. Although earlier AFL was regarded as less toxic than AFB1 (McCann *et al.*, 1975; Wong and Hsieh, 1976), recent data in fish indicate equal toxicity (Bailey *et al.*, 1994, 1998). Also, AFL was found in both maternal and fetal circulation in the perfusion, indicating that AFL reaches the fetus. Because AFL acts as a reservoir of AFB1, prolonging its lifetime in body, as it can be reconverted to AFB1 (Salhab and Edwards, 1977; Wong and Hsieh, 1976), it is probably detrimental to fetal health. Interindividual variation in the amount of formed AFL was seen, which in the case of perfusions was not related to the size of the perfused cotyledon. The fact that similar variation was seen in *in vitro* incubations gives support of a true variation in accordance with variation of placental metabolism reported earlier (Vahakangas *et al.*, 1989).

In our study, AFL was the only metabolite detected in placental models. Formation of AFL is mediated by a NADPH-dependent reductase (Salhab and Edwards, 1977). To our knowledge, only two earlier studies exist in the literature on human placental metabolism of AFB1. The first study shows that purified LO from human term placenta mediates epoxidation of AFB1 (Datta and Kulkarni, 1994). In the second study, mutagenic activity of AFB1 was increased when AFB1 was incubated with *S. typhimurium* TA100 and placental microsomal protein (Sawada *et al.*, 1993). Inhibitors of CYP enzymes indicated involvement of CYP1A and 19. However, the metabolites responsible for the increased mutagenicity were not identified. CYP1A2 and 3A4, which are mainly responsible for CYP-mediated AFB1 metabolism in the liver (Eaton and Gallagher, 1994; Gallagher *et al.*, 1994; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2002), are not functionally active in human term placenta (Hakkola *et al.*, 1996a,b). This is supported by our finding of AFL being the only AFB1 metabolite detected during perfusions and in *in vitro* incubations with human placental tissue fractions. Although AFL is mutagenic and may thus have been responsible for the increased mutagenicity in the study by Sawada *et al.* (1993), we could not detect any DNA binding in the placenta perfused with radioactive AFB1. In this perfusion, a significant amount of AFL was formed in all placentas according to the HPLC analysis.

The kinetics of AFB1 differed from that of antipyrine, which diffuses passively through the placenta (Bassily *et al.*, 1995; Brandes *et al.*, 1983; Schneider *et al.*, 1972). One reason naturally is the fast metabolism of AFB1 to AFL. However, the difference in the FM ratios with both doses of AFB1 compared to that of antipyrine may also point to other mechanisms in the transfer of AFB1 than passive diffusion. Potential interactions of AFB1 with transporter proteins in human placenta have not been studied so far. Placenta is known to express a variety of transporter proteins, including ABC transporters (p-gly/ABCB1, MRP1-3/ABCC1-3, and BCRP/ABCG2), organic anion transporters, organic cation transporters, serotonin transporter, and norepinephrine transporter (Wang *et al.*, 2007). Most abundant transporter proteins in the apical surface of syncytiotrophoplast are p-gp/ABCB1 and BCRP/ABCG2

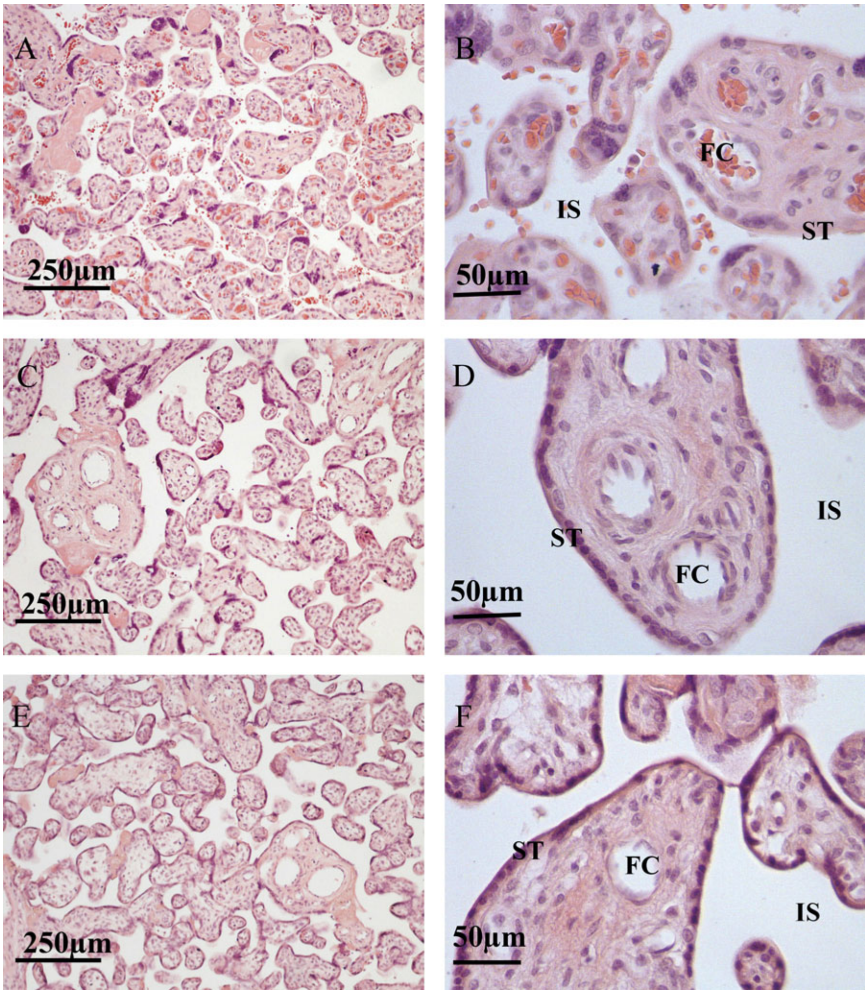


**FIG. 6.** Histological evaluation of the placental tissue using hematoxylin-eosin staining. Human placenta before perfusion with low (A) and high (B) magnification and after the perfusion with either 0.5µM AFB1 for 4 h (C and D with low and high magnification, respectively) or 5µM AFB1 for 2 h (E and F with low and high magnification, respectively). FC, fetal capillary; IS, intervillous space; ST, syncytiotrophoblast.

putatively protecting the fetus from the exposure to environmental chemicals (Behravan and Piquette-Miller, 2007). AFB1 is a probable substrate for several transporter proteins, such as BCRP, MRP1, and organic anion transporters (Loe *et al.*, 1997; Tachampa *et al.*, 2008; van Herwaarden *et al.*, 2006). Since there are high amounts of BCRP and other ABC transporters in the placenta, these transporters may have an effect on AFB1 transport through human placenta. In this study, when the FM ratio was calculated adding AFL to AFB1, it remained lower than the FM ratio of antipyrine. This may indicate a mechanism hindering to some extent the compounds in maternal circulation from entering the fetal circulation.

In conclusion, our results provide the first direct evidence of the transfer of AFB1 trough human placenta. It is possible that placental transporters have an effect on the transfer of AFB1 and will be studied in future experiments. This is also the first study to demonstrate the metabolism of AFB1 to AFL by human placental tissue. Since AFL is less mutagenic, but putatively as carcinogenic as AFB1, formation of AFL may not protect the fetus from the toxicity of AFB1.

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